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BASIC-TRANSLATIONAL REVIEW

At the Bench: Chimeric antigen receptor (CAR) T cell therapy for the treatment of B cell malignancies

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ABSTRACT

The chimeric antigen receptor (CAR) represents the epitome of cellular engineering and is one of the best examples of rational biologic design of a synthetic molecule. The CAR is a single polypeptide with modular domains, consisting of an antibody-derived targeting moiety, fused in line with T cell-derived signaling domains, allowing for T cell activation upon ligand binding. T cells expressing a CAR are able to eradicate selectively antigen-expressing tumor cells in a MHC-independent fashion. CD19, a tumor-associated antigen (TAA) present on normal B cells, as well as most B cell-derived malignancies, was an early target of this technology. Through years of experimental refinement and preclinical optimization, autologously derived CD19-targeting CAR T cells have been successfully, clinically deployed, resulting in dramatic and durable antitumor responses but not without therapy-associated toxicity. As CD19-targeted CAR T cells continue to show clinical success, work at the bench continues to be undertaken to increase further the efficacy of this therapy, while simultaneously minimizing the risk for treatment-related morbidities. In this review, we cover the history and evolution of CAR technology and its adaptation to targeting CD19. Furthermore, we discuss the future of CAR T cell therapy and the need to ask, as well as answer, critical questions as this treatment modality is being translated to the clinic. ***J. Leukoc. Biol.*** **100: 1255–1264; 2016.**

Introduction

The development and evolution of the CAR represent the culmination of advances in protein and genetic engineering, founded on a deep understanding of lymphocyte biology. Tireless work involving rigorous and thorough preclinical optimization by multiple investigative groups has led to the clinical deployment of a number of anti-TAA-targeted CARs. Nowhere else has this been more evident than in the CD19 space. To appreciate fully the elegance of the CAR molecular architecture, it is important to understand and acknowledge the biologic principles and components that underlie the foundation of this technology. In this review, we will briefly discuss the role of T cells in the control of autologous tumors and the underlying biology that allows for this control, as well as loss of immune containment. We will subsequently discuss the artificial targeting of TAAs through the use of Igs and how this phenomenon was married with the effector function of T cells yielding the CAR. Finally, we will examine the evolution of the CAR, discussing the rationale for its modular components and subsequently, discuss anti-CD19 CAR T cell preclinical data.

Abbreviations: AAPC = artificial APC, AICD = activation-induced cell death, ALL = acute lymphoblastic leukemia, B-ALL = B cell acute lymphoblastic leukemia, CAR = chimeric antigen receptor, CLL = chronic lymphocytic leukemia, CRS = cytokine release syndrome, HER2 = human epidermal growth factor receptor 2, L = ligand, mIL = murine IL, pMHC = peptide in the context of a MHC molecule, scFv = single-chain variable fragment domain,

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T CELLS, CANCER, AND THE LOSS OF TUMOR CONTAINMENT

Tumor cells often express a variety of tumor-exclusive, mutation-derived neoantigens, a number of which can be recognized by the adaptive immune system [1, 2]. The consequence of the immune system recognizing these TSAs has been shown extensively, most notably, by the existence of TILs, whose presence has correlated with improved prognosis in a number of malignancies [3]. Of these TILs, tumor antigen-specific T cells have been shown to play a major role in tumor control. Pioneering work by Rosenberg et al. [4], involving TIL isolation, followed by ex vivo expansion and adoptive transfer back into patients, resulted in demonstrable control of autologous tumors [5, 6]. Importantly, it was shown that these adoptively transferred, tumor-specific T cells were able to localize to the tumor postinfusion, thereby presumably impacting their effector function [7]. Major limitations of this adoptive immunotherapy platform are dependence on the presence and isolation of tumor-specific T cells, which could be cumbersome. Clinical use

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of this technology is further complicated by the down-regulation of antigen-processing machinery by tumor cells, an established mechanism used by malignancies to evade T cell-mediated elimination [8].

> **TSA:** *An antigen that is exclusively found in or significantly overexpressed in cancer cells compared with normal tissue.*

Additionally, a number of tumors are able to perpetuate an immunosuppressive and immunotolerant microenvironment through interactions with nonmalignant stromal elements, such as myeloid-derived suppressor cells, endothelial cells, regulatory T cells, and Th cells, leading to decreased effector function and subsequent exhaustion of infiltrating tumor-specific T cells [9]. These described mechanisms eventually lead to loss of immune-mediated tumor containment and unbridled growth of the malignancy.

> **Clinical Question:** *Could pharmacological agents be used to increase the immunogenicity of tumors through up-regulation neo-antigens, TSAs, or tumor–cell antigen-presenting machinery, all leading to enhanced tumor immunogenicity?*

THE BIOLOGY OF T CELL-MEDIATED KILLING

Physiologically, malignancy-specific T cell clones are primed to eradicate tumor cells by prior stimulation through APCs, most commonly, dendritic cells. The T cell–APC interface is complex and involves the interaction of receptors on either cell with their cognate ligands on the other (see **Fig. 1**). Experimental evidence has established that for optimal T cell activation, at least 2 particular types of receptor–ligand interactions need to occur, observations that led to the concept of a 2-signal model of T cell activation [10–13]. The first signal, commonly referred to as signal 1, is delivered through the interaction of a clonal TCR complex and its accessory molecule, CD4 or CD8, with a TCR-specific pMHC on an APC. This interaction leads to the initiation of downstream signaling via the TCR. In isolation, signal 1 is incapable of activating a T cell and when delivered alone, can lead to AICD and/or an unresponsive state, called anergy [14, 15]. For T cell activation and subsequent proliferation to be realized, APCs need to deliver a secondary costimulatory signal, signal 2. The 2 main receptor families involved in mediating costimulation on T cells are the following: 1) the CD28 family, including CD28 and ICOS, interacting with their cognate ligands CD80/CD86 and B7-H2, respectively, and 2) the TNFRSF, including 4-1BB, CD27, and OX40, interacting with 4-1BBL, CD70 (CD27L), and OX40L, respectively. Whereas the interaction of the TCR/pMHC

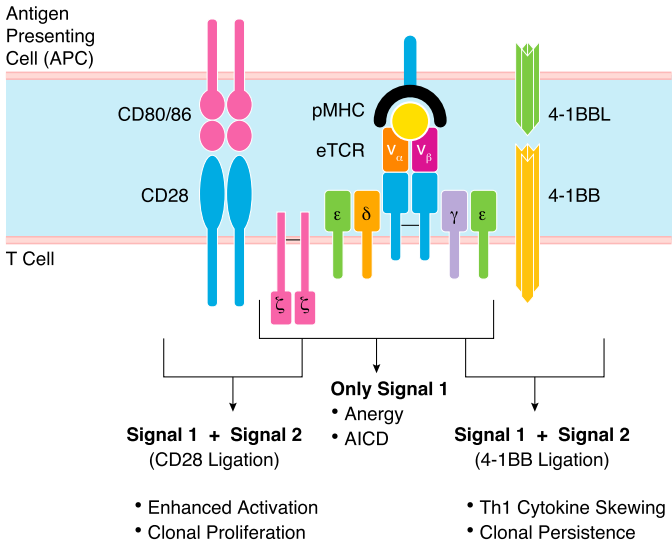

Figure 1. The APC–T cell interface. Ligation of a pMHC complex by an endogenous TCR delivers signal 1 to the T cell. In the absence of signal 2 via ligation of a costimulatory receptor (either CD28 or 4-1BB, as shown above), T cells can become anergic or undergo AICD. Concomitant engagement of T cell-bound CD28 or 4-1BB delivers signal 2, leading to optimal T cell activation. For simplicity, accessory molecules CD4 or CD8 on T cells are not shown. eTCR, Endogenous TCR; Vα, variable region of the TCR α-chain; Vβ, variable region of the TCR β-chain; γ/δ/ε/ζ, CD3-γ/δ/ε/ζ-chains.

ensures the potential activation of a peptide-specific T cell by an APC, the delivery of costimulatory signals can have varying consequences on T cell fate, including survival, effector function, and establishment of memory [16]. Once a T cell has received signals 1 and 2, it is primed and activated (Fig. 1). In the case of a tumor-specific T cell, once the TCR ligates its cognate TSA peptide displayed within an MHC, the T cell initiates a cytolytic cascade that results in the death of the target cell.

> **Clinical Question:** *Is there a particular combination of signals 1 and 2 that leads to optimal T cell activation, and could this knowledge be translated with synthetic biology to develop improved therapeutic agents?*

THE THERAPEUTIC EXPLOITATION OF SURFACE-EXPOSED TAAs

The immunogenicity of TSAs is dependent on their ability to be processed by the tumor cell's antigen-presentation machinery, for this processed protein to be mounted and displayed on an MHC, and for this pMHC complex to be recognized by an appropriate TCR. There exist, however, wild-type extracellular proteins that are expressed on both normal as well as transformed cells. In the latter, expression could be lineage dependent and normal. Given their presence on both normal and malignant cells, these extracellular proteins can be considered TAAs.

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TAA = tumor-associated antigen, tEGFR = truncated human epidermal growth factor receptor, TIL = tumor-infiltrating lymphocyte, TNFRSF = TNFR superfamily, TNP = 2,4,6-trinitrophenyl, TSA = tumor-specific antigen, V_H = variable regions of the heavy chain, V_L = variable regions of the light chain

TAA: *An antigen relatively restricted to tumor cells but present to a lesser extent on normal tissue.*

Native-conformation TAAs are not normally targetable by endogenous T cells and when processed, are less likely to be immunogenic secondary to self-tolerance, given their presence on nontransformed cells. However, these TAAs can be targeted with exogenously derived Igs, which have an antigen-recognizing domain and unlike that of a TCR, can recognize surface-exposed, unprocessed antigens in an MHC-independent manner.

Clinical Questions: *Do levels of expression or densities of extracellular TAAs on tumor cells dictate the susceptibilities of these cells to targeted therapies? Can strategies aimed at increasing the level of these antigens improve clinical outcomes with targeted agents?*

THE DEVELOPMENT OF THE CARs

Creation of the CAR evolved out of the idea to confer T cells with the ability to recognize antigens in an antibody-dependent, MHC-independent manner through the genetic engraftment of the antigen-recognizing domain of an Ig into a T cell [17]. This approach was deemed possible, given the structural similarities that exist between the antigen-binding sites of a TCR and the antigen-recognizing component of an Ig Fab (fragment antigen binding) [18]. Seminal work by Eshhar et al. [17] in 1989 described the splicing of the V_H and V_L chains of SP6, an antibody specific to TNP, to the constant region of the TCR α- or β-chains of a cytotoxic T cell hybridoma (**Fig. 2**). The group reported that stimulation of a chimeric receptor bearing T cell hybridomas with TNP-bearing cells led to production of IL-2 and the killing of the target cells, demonstrating that the chimeric TCR was, in fact, biologically functional. This concept of a chimeric Ig–TCR was corroborated by Goverman et al. [19] in 1990. The group demonstrated that such a molecule, generated through the fusion of a phosphorylcholine-specific antibody's V_H (which in this antibody, provides most of the antigen recognition for the hapten) to either the TCR α- or β-chains, was functionally active.

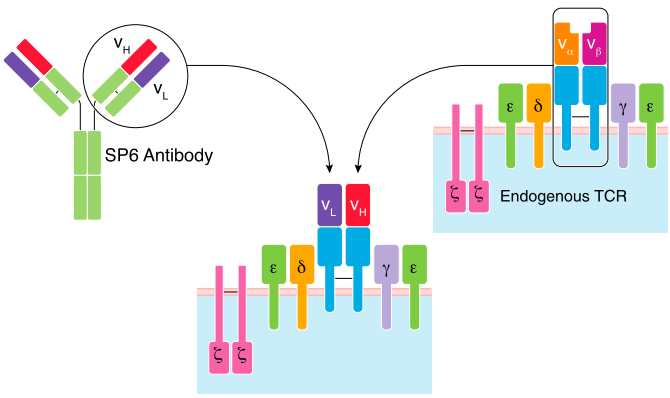

Figure 2. The first T cell-based chimeric antigen receptor. Eshhar and coworkers [17] successfully engrafted the V_H and V_L chains of SP6, an antibody specific to TNP, to the constant region of the TCR α- or β-chains of a cytotoxic T cell hybridoma.

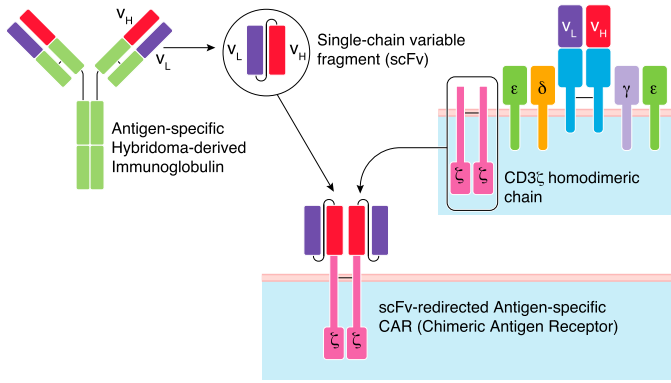

Figure 3. The first-generation CAR. The V_L and V_H chains of an antigen-specific Ig were combined by a flexible linker making an scFv, which was then fused in line with the CD3ζ signaling domain. An alternative design included fusing the scFv to the FcRγ chain, a homodimeric transmembrane molecule with similar structure and signaling signature to CD3ζ.

CAR, THE FIRST GENERATION

In 1993, the design of the CAR (then termed T-body) was improved upon by integrating the targeting and signaling motifs into one single polypeptide chain [20, 21] (**Fig. 3**). In one of these initial iterations [20], the VL and V_H chains of MOv18, an anti-ovarian carcinoma antibody targeting folate receptor α, were combined by a flexible linker making a scFv. This scFv was then fused in line with the FcRγ chain, a homodimeric transmembrane molecule with a similar structure and signaling signature to CD3ζ. With the use of contemporary gene-transfer technology that allowed for the integration of genetic material into primary T cells using gammaretroviral-based transduction [22], this construct was successfully introduced into human T cells obtained from melanoma-derived TILs. In vitro assays demonstrated that these CAR T cells were functional, as they were able to kill specifically tumor APCs and produce GM-CSF in response to CAR stimulation. In the other reported version of the single-chain polypeptide CAR [23], the investigators generated an anti-HER2 scFv and fused this in line with CD3ζ, a molecule that naturally exists as a homodimer, allowing for potential dimerization of the artificial construct. CTL hybridomas expressing this chimeric protein were able to kill Neu/HER2-expressing cells in cocultures and secrete IL-2 in an antigen-specific, non-MHC-dependent manner. These constructs effectively demonstrated that T cell effector function can be redirected to a prespecified-native, unprocessed, surface-exposed antigen through the use of a single-chain modularly designed chimeric protein consisting of an antibody-derived targeting moiety, fused to a T cell-derived intracellular signaling domain.

scFv: *A single-chain synthetic construct generated by fusing the antigen-binding domains of the heavy and light chains of an antibody with a flexible protein linker.*

The in vivo assessment of CAR-modified cells to eradicate disease soon followed. Moritz et al. [23] described a CAR consisting of a human ERBB2-recognizing scFv fused in line with the transmembrane and cytoplasmic domains of the CD3ζ chain. Unlike the earlier reported versions of the CARs that fused the scFv directly to the CD3ζ or FcR γ chain, this group's design interspaced the targeting and signaling moieties with a flexible hinge motif derived from the membrane proximal portion of CD8. This chimeric protein was integrated into CI96, a murine cytotoxic T cell clone, using retroviral gene transfer. The group demonstrated that CAR^+ CTLs had increased secretion of IFN-γ, as well as cytotoxic activity in an antigen-specific, non-MHC-dependent manner. In vivo modeling used BALB/c nude mice inoculated subcutaneously with NIH-3T3 cells expressing ERBB2, followed by a single injection of 10^7 parental/CAR^+ CTLs on d 4 or 5, supported by 3 daily injections of exogenous IL-2 on d 4–6. In these studies, CAR^+ CTLs were able to infiltrate and retard but not eradicate tumor growth compared with parental, non-transduced CTLs. Follow-up work by the group using the same construct in an immunocompetent BALB/c mouse model demonstrated that complete tumor regression could be achieved via 5 daily intratumoral injections of CAR^+ syngeneic T cells [24]. This work, using primary murine lymphocytes, demonstrated that CAR T cells had biologic activity when engrafted in autologous effector cells but were unable to bring about complete tumor eradication in the absence of direct administration into the tumor bed. These and other early constructs [17, 25], given their use of a singular intracellular signaling domain providing signal 1 on chimeric protein ligation, made up the first generation of CARs.

FIRST-GENERATION CARS: THE CD19 EXPERIENCE

CD19 as a TAA

CD19 is a 95 kDa transmembrane glycoprotein that is found on most cells of the B lineage, excluding terminally differentiated plasma cells. CD19 is not present on pluripotent hematopoietic stem cells [26] and is expressed on most B cell-derived leukemias and lymphomas [27]. Given the restrictive expression pattern of this antigen, CD19-positive malignancies became an early target for antibody-mediated immunotherapy [28] and subsequently, CAR-mediated targeting.

The anti-CD19 CAR

In 2003, we described the use of CD19-targeted CAR T cells in the treatment of human B cell malignancy xenografts [29]. The CAR construct used a human CD19-specific scFv derived from the SJ25C1 hybridoma [27, 30], fused in line with the hinge and transmembrane domain of human CD8-α and the cytoplasmic domain of the CD3ζ: the construct was termed 19z1. As assessed by chromium release assay, primary human T cells retrovirally transduced with this 19z1 were able kill, in an antigen-specific manner, $CD19^+$ human Burkitt lymphoma (Raji and Daudi) and BALL (Sup-B15 and NALM-6) cell lines. Additionally, it was shown that 19z1 T cells derived from CLL patients of various clinical stages and prior chemotherapy treatment profiles, led to the in vitro lysis of autologous CLL cells, demonstrating the feasibility of using an autologous CAR T cell platform for the treatment of $CD19^+$ B cell malignancies.

Several groups [25, 31, 32] demonstrated that a CAR solely incorporating a CD3ζ signaling domain was insufficient to bring about complete T cell activation and robust expansion of modified primary T cells. To overcome this limitation, we generated CD19/$CD80^+$ NIH-3T3 AAPCs. These AAPCs were capable of ligating the CAR and simultaneously delivering a costimulatory signal to the T cells via CD80 interacting with the CD28 costimulatory receptor. In vitro proliferation assays using these AAPCs as stimulators demonstrated that 19z1 T cells expanded best in the presence of these AAPCs, as well as in the presence of exogenous IL-15 compared with conditions omitting CD80 or substituting IL-2 for IL-15. Importantly, costimulation of 19z1 T cells under these conditions led to retention of their cytotoxicity on restimulation with antigen and resulted in selective enrichment of CAR^+ T cells. These findings implicated the importance of costimulatory signals in bringing about optimal CAR T cell activation, demonstrating that combinatorial signals via CD3ζ and accessory molecules needed to be present for the full effector function of these genetically engineered T cells to be realized.

AAPC: *Cells genetically engineered to express a target antigen of choice, as well as membrane-bound costimulatory molecules or cytokines.*

To assess the contribution of costimulation to the ability of 19z1 T cells to eradicate disease in vivo, we engrafted SCID-Beige mice with Raji (which express CD80 and CD86) and NALM-6 (which lack CD80 and CD86 costimulatory molecules) cells. A single treatment of Raji tumor-bearing mice with 19z1 T cells led to 50% long-term survival and was further increased to 75% after a second infusion of CAR T cells. Whereas treatment with 19z1 T cells was able to bring about durable, long-term survival of at least one-half of Raji tumor-bearing mice, the same was not the case for the NALM-6-engrafted SCID-Beige. In this model, the cohort receiving 19z1 T cells had a prolonged median survival, but all animals eventually succumbed to disease. However, mice engrafted with NALM-6, transduced with CD80, were afforded 40% long-term survival when treated with 19mz1 T cells, demonstrating the critical role of in vivo costimulation in enhancing in vivo CAR T cell effector function.

Given that most tumors do not express costimulatory molecules, other means of enhancing the effector function of first-generation CD19 targeting CAR T cells were attempted. Cheadle et al. [33] demonstrated that preconditioning with intraperitoneal cyclophosphamide led to improved long-term survival in 19z1-treated Raji tumor-bearing SCID-Beige mice. The mechanism of action mediated by this cytoreductive therapy was,

in some part, related to its ability to decrease tumor burden before the infusion of CAR T cells in this xenograft model. The role of cyclophosphamide conditioning was further elucidated in a syngeneic mouse model [34]. With the use of immunocompetent human CD19 transgenic mice engrafted with syngeneic human CD19-expressing EL4 tumors (a thymoma-derived cell line), we showed that cyclophosphamide conditioning of tumor-bearing mice led to a decrease in T regulatory cells and a transient increase in IL-12 and IFN-γ. Establishment of long-term survival in the setting of treatment with 19mz1 (a syngeneic, anti-CD19, first-generation CAR with a murine CD3ζ signaling domain) T cells was dependent on cyclophosphamide preconditioning and resulted in both tumor eradication and B cell aplasia—the latter a surrogate of continued CAR T cell activity and a manifestation of the "on-target, off-tumor" effect. We further described the generation of a novel chimeric vector containing 19mz1 in line with an internal ribosome entry site and a gene encoding a fused heterodimeric mIL-12, allowing for T cells engrafted with this construct to express the 19mz1 CAR and constitutively secrete mIL-12. Tumor-bearing mice treated with 19mz1/mIL-12-expressing T cells developed B cell aplasia and displayed long-term survival in the absence of cyclophosphamide preconditioning—outcomes that were dependent on $CD4^+$ and $CD8^+$ T cell subsets. Results of this work demonstrated that mIL-12-secreting, first-generation CAR T cells, compared with 19mz1, had an augmented function and could bring about eradication of systemic disease, independent of cyclophosphamide preconditioning.

On-target, Off-tumor: *A toxicity arising from the killing of target-positive, nontumor cells by CAR T cells.*

Clinical Question: *Should tolerable on-target, off-tumor toxicities be used as an indicator for dose adjustments of CAR T cells?*

The above results demonstrated that optimal expansion and in vivo efficacy of first-generation CAR T cells were dependent on the presence of costimulatory molecules on target cells (a phenomenon not shared by many tumor cells), cytokine support, or preconditioning with cytotoxic chemotherapy. Given this limitation on external factors, an approach to augment CAR T function through further protein engineering was undertaken, ushering in the era of the second-generation CAR.

Clinical Question: *What is the optimal preconditioning regimen to allow for CAR T cell engraftment in humans, and is such a regimen tumor specific?*

SECOND-GENERATION CARS: CD28 AND 4-1BB

Biologic rationale for signal 2

With the drawing from lymphocyte biology on the requirement costimulation (signal 2) in the setting of TCR-mediated T cell activation (signal 1), attempts were made to incorporate the signaling domains of T cell-based costimulatory molecules in cis with that of CD3ζ (**Fig. 4**). Such a CAR would be capable of delivering both signals 1 and 2 upon ligation of target antigen (**Fig. 5**). The frontrunner candidate molecules were CD28 and 4-1BB, as these had become appreciated mediators of costimulatory stimuli. Constructs incorporating these costimulatory molecules in tandem with CD3ζ became known as second-generation CARs.

Second-Generation CAR: *A CAR with two tandem intracellular signaling domains—one commonly derived from CD3ζ and the other from a costimulatory molecule.*

CD28

CD28 is a 44 kDa, disulfide-linked homodimer belonging to the Ig superfamily and in humans, is constitutively expressed on a majority of resting $CD4^+$ and $CD8^+$ T cells [35]. The natural ligands for CD28 include the costimulatory ligands CD80 and CD86. Ligation of CD28 enhances CD3-mediated activation [36] and in the context of antigen-dependent T cell stimulation, supports clonal expansion [37] and augments T cell survival through up-regulation of Bcl-xL [38].

The first successful attempt at creating a second-generation CAR was described by Finney et al. [38] in 1998. This group developed a chimeric receptor consisting of an anti-CD33 scFv fused in line with the membrane-proximal, -transmembrane, and -intracellular domains of CD28, followed distally by the CD3ζ chain. It was demonstrated that this CAR, when integrated into Jurkat cells via electroporation, led to increased antigen-stimulated IL-2 production, a biomarker of T cell costimulation. In 2002, Maher et al. [39] reported similar findings when describing a prostate-specific membrane antigen-targeting CAR, incorporating tandem CD28 and CD3ζ signaling domains. In vitro, these CAR T cells displayed antigen-specific lytic functions and enhanced IL-2 secretion on stimulation and were capable of robust expansion upon ligation with target antigen in the absence of costimulatory ligands. Interestingly, both studies demonstrated the

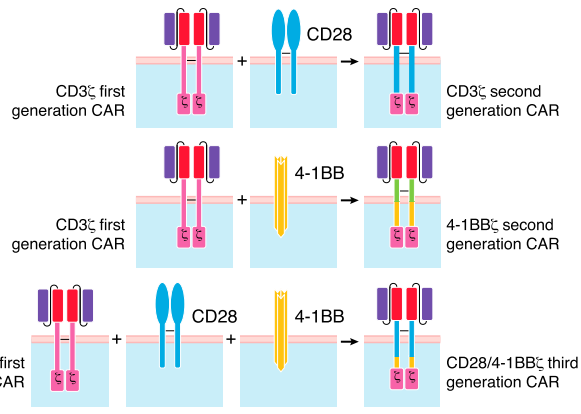

Figure 4. The evolution of the CAR. First-generation CARs augmented by inclusion of CD28 and 4-1BB intracellular signaling domains.

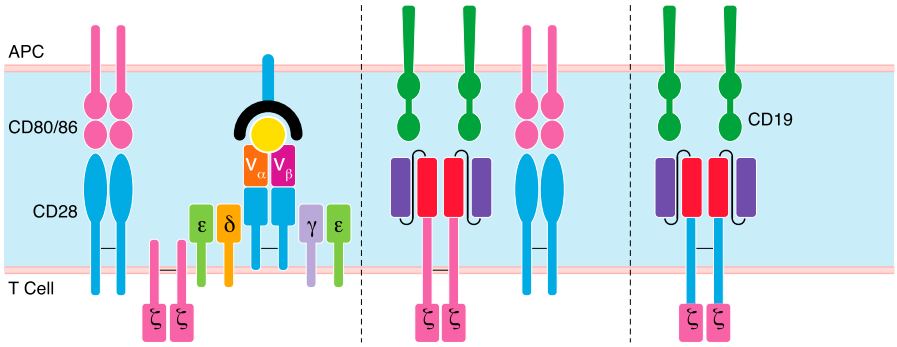

Figure 5. The second-generation CAR provides optimal signaling.

importance of signaling domain orientation, as placement of the CD28 signaling domain distal to that of CD3ζ abrogated the enhanced function of the CAR.

CD28-based anti-CD19 second-generation CARs

Work by Kowolik et al. [40] in 2006 demonstrated that anti-CD19 CAR T cells, containing both the CD28 and CD3ζ signaling moieties (second-generation CAR T cells) compared with only the CD3ζ (first-generation CAR T cells), were able to produce IL-2 and proliferate in vitro in the absence of an exogenous source of this cytokine on stimulation by $CD19^+$ target cells. Quantitative RT-PCR revealed that target-stimulated, second-generation CAR T cells markedly up-regulated Bcl-XL, a finding not observed in first-generation CAR T cells. With the use of NOD/SCID mice, engrafted with $CD19^+$ *hRluc*$^+$ (Renilla luciferase) Daudi tumor cells, it was shown that second-generation CAR T cells led to tumor eradication, as assessed by bioluminescence. Importantly, it was shown that in tumor-bearing mice, second-generation CAR T cells persisted for >30 d, whereas first-generation CAR T cells became undetectable after 10. This demonstrated the importance of a costimulatory signal in enhancing in vivo persistence of CAR T cells.

In 2007, we [41] described the construction and comparison of a number second-generation anti-CD19 CARs containing a variety of costimulatory domains in tandem with the CD3ζ signaling domain: CD28-CD3ζ, DAP10-CD3ζ, 4-1BB-CD3ζ, and OX40-CD3ζ. All constructs similarly killed NALM-6 tumors in vitro. On assessment of proliferation and cytokine production, the only second-generation CAR T cells that were capable of expanding and secreting IL-2 and IFN-γ when stimulated on $CD19^+/CD80^-$ NIH-3T3 AAPCs were those with the CD28–CD3ζ signaling domains. With the comparison of these second-generation CARs to a CD3ζ-based, first-generation CAR, both constructs had an identical efficacy in providing long-term survival to SCID-Beige mice engrafted with Raji tumor cells—an outcome attributed to the presence of costimulatory molecules on the tumor cells. In the NALM-6 xenograft model, long-term survival was achieved only with the second-generation CAR and only in the setting of 3 daily intravenous treatments. These results implicated the importance of the costimulatory signal in enhancing T cell effector function and demonstrated that integration of this signaling motif into the CAR construct led to antigen-dependent enhancements in proliferation, cytokine secretion, in vivo persistence, and eradication of costimulatory ligand (CD80, CD86)-negative tumor cells.

4-1BB

4-1BB is a 30 kD type I glycoprotein and a member of the TNFRSF. Its cognate ligand is 4-1BBL, which is usually expressed on activated APCs. 4-1BB is inducible and primarily found on antigen-activated $CD4^+$ and $CD8^+$ T cells. In a CD28-independent manner, ligation of 4-1BB mediates T cell proliferation, production of predominantly Th1 cytokines, augmentation of cytotoxic capabilities, and T cell survival through up-regulation of anti-apoptotic protein Bcl-XL [42].

In 2004, Imai et al. [43] were the first to described a 4-1BB-containing, second-generation CAR targeting CD19, using an scFv derived from the FMC63 hybridoma [44]. The group fused this scFv to the CD8α hinge and transmembrane domains, in line with the intracellular domain of 4-1BB in tandem with that of CD3ζ. Compared with first-generation anti-CD19 T cells, second-generation, 4-1BB-based T cells had enhanced cytotoxicity against primary ALL cells and increased production of IL-2 when cocultured with $CD19^+$ target cells.

In 2009, Milone et al. [45] compared CD19 targeted CD28- and 4-1BB-based, second-generation CAR T cells. 4-1BB-based, second-generation CAR T cells produced less IL-4 and IL-10, both Th2 cytokines, on stimulation by antigen-positive stimulator cells compared with CD28-based, second-generation and CD3ζ-based, first-generation CAR T cells. It was further demonstrated using NOD/SCID-γ mice, engrafted with primary pre-B-ALL cells, that the 4-1BB-based, second-generation CAR T cell provided the longest leukemia-free survival. One possible reason for this enhancement in disease control was attributable to the ability of the 4-1BB-based, second-generation CAR T cells to undergo antigen-independent, in vitro proliferation after initial T cell stimulation, potentially

leading to augmented in vivo expansion and persistence, resulting in longer disease-free periods.

THIRD-GENERATION CARS: COMBINING COSTIMULATORY DOMAINS

The work previously described by Milone et al. [45] also assessed the use of integrating 2 costimulatory signaling domains, CD28 and 4-1BB, in line with CD3ζ, into an anti-CD19-targeting CAR. Such constructs became termed third-generation CARs. The group demonstrated that this CAR was functionally active and the most potent of its constructs, as assessed by in vitro and in vivo assays, but was unable to show that this enhanced function translated into a statistically significant survival difference compared with its 4-1BB-based, second-generation CAR T cell.

Third-Generation CAR: *A CAR with 3 tandem intracellular signaling domains—1 derived from CD3ζ and the other 2 from a costimulatory molecule.*

A comparison of second- and third-generation, CD19-targeted CAR T cells was made by Kochenderfer et al. [46] in 2009. The group demonstrated in in vitro studies using $CD19^+$ target cells (including CLL) that its CD28-based, second-generation CAR T cells produced more IFN-γ and that a higher percentage of these CAR T cells produced IL-2 compared with its CD28/4-1BB/CD3ζ-based, third-generation CAR T cell. These findings led the group to pursue clinical trials with its second-generation CAR T cell.

However, work by Tammana et al. [47] demonstrated the superiority of umbilical cord-blood cells containing a third-generation, anti-CD19 CAR, incorporating signaling domains of CD28, 4-1BB, and CD3ζ compared with second-generation CARs containing CD3ζ with CD28 or 4-1BB. With the use of in vivo studies to evaluate the superiority of either generation, the group showed that its third-generation CAR T cells conferred prolonged survival in both intraperitoneal and systematic models of xenografted B cell tumors.

The variability in preclinical in vitro and in vivo results reported by the different groups comparing second- and third-generation, CD19-targeted CAR T cells leaves the question as to which is better unanswered. The inconsistencies in the results reported are further complicated by the differences in the materials and methods used by the various investigative groups, including the choice of scFv fragments used (i.e., from what hybridomas they were derived), the architectural makeup of the receptor (CD28 vs. 4-1BB), and the method of construct delivery into cells (see **Table 1**).

TABLE 1. Vectors used for CAR construct delivery into T cells

Replication-Incompetent Retroviruses
Gammaretroviral vectors
Efficient genomic integration with stable gene expression
Inexpensive
Transduce only dividing cells
Risk for insertional mutagenesis when standard dual LTR enhancer–promoter sequence-based viruses are used, as compared to SIN (self-inactivating) variants
Lentiviral vectors
Can transduce nondividing cells
Reduced risk for insertional mutagenesis
Transposable elements (transposon/transposase)
Sleeping Beauty and PiggyBac
Less immunogenic than retroviral vectors
Vector delivered via electroporation
Inexpensive

Clinical Questions: *Is there a need to compare clinically the various second-generation, as well as third-generation CARs with one another? How can such a comparison be made across such disparate products?*

THE ARMORED CAR AND CHECKPOINT BLOCKADE: THE ERA BEYOND THE SECOND AND THIRD GENERATION

As anti-CD19 CAR T cells continue to demonstrate clinical success, preclinical work is now focused on further improving the efficacy of this technology (**Fig. 6**). The armored CAR represents the next step in design evolution. In this generation, augmentation on T cell function is moved away from the CAR construct; rather, it is focused on the genetic inclusion of factors that can act in trans, affecting not only the genetically engineered cell but also, allied cells within hostile tumor microenvironments, such as those seen in bulky B cell malignancies and solid tumors.

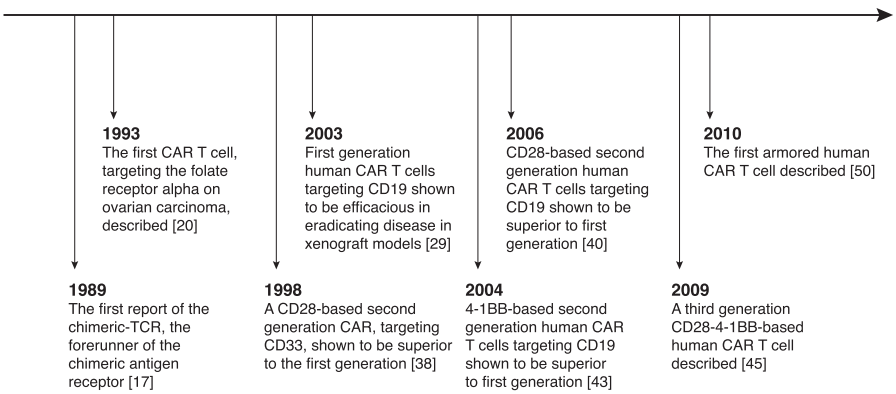

Figure 6. Timeline of CAR T cell development and optimization.

Armored CAR: *A second-generation CAR further engineered to express constitutively a membrane-bound or secretable immunomodulatory molecule.*

Given the exclusion of these "armors" from the control of the CAR, their expression is not tied into antigen ligation but is constitutive. Our laboratory has pioneered 2 of these armored anti-CD19 CARs—1 that secretes IL-12 [34, 48] and the other that constitutively expresses CD40L [49]. Other transactivating factors that have been described in armored anti-CD19 CARs include IL-15 [50] and 4-1BBL [51]. In the preclinical setting, CAR T cells expressing these armaments show an enhanced ability to kill target cells and improve survival in tumor-engrafted animals. Given the track record of translating bench-based CAR technology to the clinic, these "armored" CARs will, no doubt, make their way to the clinic at some point in the future.

Clinical Questions: *Will the enhanced function of armored CAR T cells translate into improved clinical outcomes? Should armored CARs supplant the use of second- and third-generation CARs in the clinic?*

CAR T cells, despite their genetic modification, are still susceptible to cellular exhaustion and effector dysfunction, most likely as a result of interacting with inhibitory molecules on tumor cells or within the tumor microenvironment. These detrimental phenotypes will become critically important as CAR T cell therapy makes its way into the treatment of solid malignancies—cancers that are known for their immunosuppressive qualities. Exciting preclinical work has demonstrated that checkpoint blockade augments CAR T cell function, leading to enhanced clearance of solid tumors, with responses to the combined therapies exceeding those of either therapy alone [52].

Clinical Questions: *Will checkpoint blockade clinically improve CAR T cell function? Which is the more tolerable and efficacious axis to interrupt: programmed death 1 or CTLA-4? Should checkpoint blockade be considered as a standard addition, as with preconditioning regimens, in future CAR T cell trials?*

BENCH-TO-BEDSIDE DISCORDANCE: THE CASE OF CRS

The clinical success of second-generation anti-CD19 CAR T cells, particularly in the treatment of relapsed/refractory B-ALL, has come at a price, with the occurrence of a mostly transient yet clinically relevant toxicity known as CRS (see ref. [53] for a more in-depth description of CRS). Unlike therapy-related B cell aplasias, which are evident in syngeneic mouse models, CRS has no convincingly clear preclinical or experimental parallels, making elucidation and scientific clarification of this phenomenon extremely difficult. Given CRS, there has been a move in CAR design to incorporate elimination genes or inducible apoptotic switches. Huang et al. [54] describe the incorporation of CD20 into a CD19-targeted CAR, allowing for elimination using rituximab, a clinically approved anti-CD20 antibody. A similar approach incorporating a tEGFR into anti-CD19 CARs has also been described [55]. This tEGFR lacks an N-terminal ligand-binding domain but retains its binding epitope for cetuximab, a U.S. Food and Drug Administration-approved anti-EGFR biologic. Another elimination gene that has been integrated into anti-CD19 CARs is inducible caspase-9, which in the presence of the chemical inducer of dimerization, AP20187, leads to elimination of transgenic T cells [50]. Clinical trials with CAR T cells expressing these "suicide genes" will determine whether these constructs will be able to curb CRS.

Clinical Questions: *What is the etiology of CRS, and will incorporation of suicide genes in CAR T cells improve their safety profile? Is CRS tumor specific, and if not, why?*

CONCLUSION

The CAR affords genetically engineered T cells the ability to eradicate target-bearing tumor cells in an MHC-independent manner. Multiple investigative groups have created and optimized architecturally distinct CARs with variations in the hybridomas from which their scFvs are derived, the nature of the spacer/hinge, and the tandem signaling domains, as well as the route of construct delivery into T cells. Despite these variations, clinical implementation of this technology by these varying groups has resulted in the successful treatment of $CD19^+$ relapsed/refractory B cell malignancies, particularly in the setting of B-ALL. With this success also comes toxicity and in some cases, the enrichment of antigen-loss variants [56, 57]. Current work at the bench is focused on further augmenting CAR T cell efficacy through further genetic modification of the T cell product, as well as through the rational combination of this technology with standard antineoplastic therapies, including pharmacological agents [58] and checkpoint blockade [52].

AUTHORSHIP

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